Short Technical Reports

Multiplex Manager 1.0: a cross-platform computer program that plans and optimizes multiplex PCR

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Multiplex Manager 1.0 is a user-friendly cross-platform program that designs efficient combinations of existing genetic marker loci into multiplex polymerase chain reactions and optimizes using prior marker information. The program has the flexibility to solve two design problems: combining all markers into the smallest number of reactions, or alternatively, selecting a subset from many available markers to design an efficient and robust multiplex. Our program minimizes the number of reactions, the genetic linkage, and the difference in annealing temperature. At the same time it maximizes the spacing between markers, the heterozygosity, and the number of alleles. The final output provides easily interpreted and informative graphical representations of reactions, as well as the option of manually editing final reactions. Multiplex Manager 1.0 is freely available at www.multiplexmanager.com.

Introduction

Multiplex PCR, where multiple genetic loci are amplified in a single reaction, is a technique increasingly employed to dramatically improve the time and cost efficiency of genetic studies. However, designing a successful multiplex reaction is often a time-consuming and difficult process, requiring the simultaneous optimization of multiple factors, such as allele size range, annealing temperatures, complementarity of primers, heterozygosity, number of alleles, and genetic linkage. In addition, there are often extrinsic factors to consider, such as known artifacts associated with a particular method of fragment analysis. Most programs currently available for planning multiplex PCR are concerned with de novo primer design from flanking sequence (1–3), or only address a single variable involved with planning a multiplex, such as primer complementarity in the program AutoDimer (4). Multiplex Manager solves a different problem: evaluating a variety of factors in order to create novel multiplex reactions using previously published primer sequences. There are several benefits in designing multiplexes for new combinations of previously published markers: researchers do not have to invest time in marker development; they can utilize previously existing information (e.g., heterozygosity) to ensure suitability of the selected markers; and they can streamline their existing molecular techniques while still maintaining continuity of information. We have developed Multiplex Manager 1.0 to meet the growing demand for a program that facilitates effective and efficient multiplex planning for previously published genetic markers as well as utilizing the available prior information.

Materials and methods

In Multiplex Manager, users enter information about their markers into the Marker Data window depicted in Figure 1A. The minimum data requirements to execute Multiplex Manager are entries in the marker name and allele size range fields. However, to obtain the most successful multiplex, we recommend providing as much information as possible in the Marker Data window. For example, minimizing the difference in primer annealing temperatures simplifies the task of achieving uniform amplification across loci within a multiplex, and thus makes a more reliable and robust PCR reaction (5). Users can supply empirical information about the primer annealing temperatures. Alternatively, if users do not have an empirical estimate of annealing temperature for each marker, Multiplex Manager can estimate annealing temperature based on the thermodynamic parameters of neighboring bases in the primer sequences (6). After entering all available information about the genetic markers into the Marker Data window, users move to the Dyes and Options window to further define the nature of the optimization required.

One of the most crucial elements of efficient multiplex PCR is the ability to differentiate markers with the same allele size range, usually using fluorescent primer labeling. Users specify which fluorescent dyes are to be used in the Dyes and Options window depicted in Figure 1B. Users can edit and prioritize the four default dyes (6-FAM, VIC, NED, and PET) or add new dyes with an appropriate color in the first column. Users can enter known artifacts that may be associated with certain dyes in the second column. The program will not assign a marker to a particular dye if the allele size range overlaps with the specified artifact. The third column allows the enforcement of a marker to be labeled with a particular dye.

At the bottom of the Dyes and Options window are the Control Parameters, which the program uses to generate solutions. Researchers must specify which of the following two design scenarios is appropriate for their data. Scenario one is when a small number of markers must be combined into the smallest number of reactions. Scenario two is when there is a large database of available markers from which a smaller subset must be chosen. Scenario two is common for organisms that have a large number of genetic markers available, such as humans (7), Atlantic salmon (*Salmo salar*) (8), the fruit fly (*Drosophila melanogaster*) (9), rice (*Oryza sativa*) (10), or cotton (*Gossypium hirsutum*) (11).

Users can select which criteria they wish to apply when generating a solution

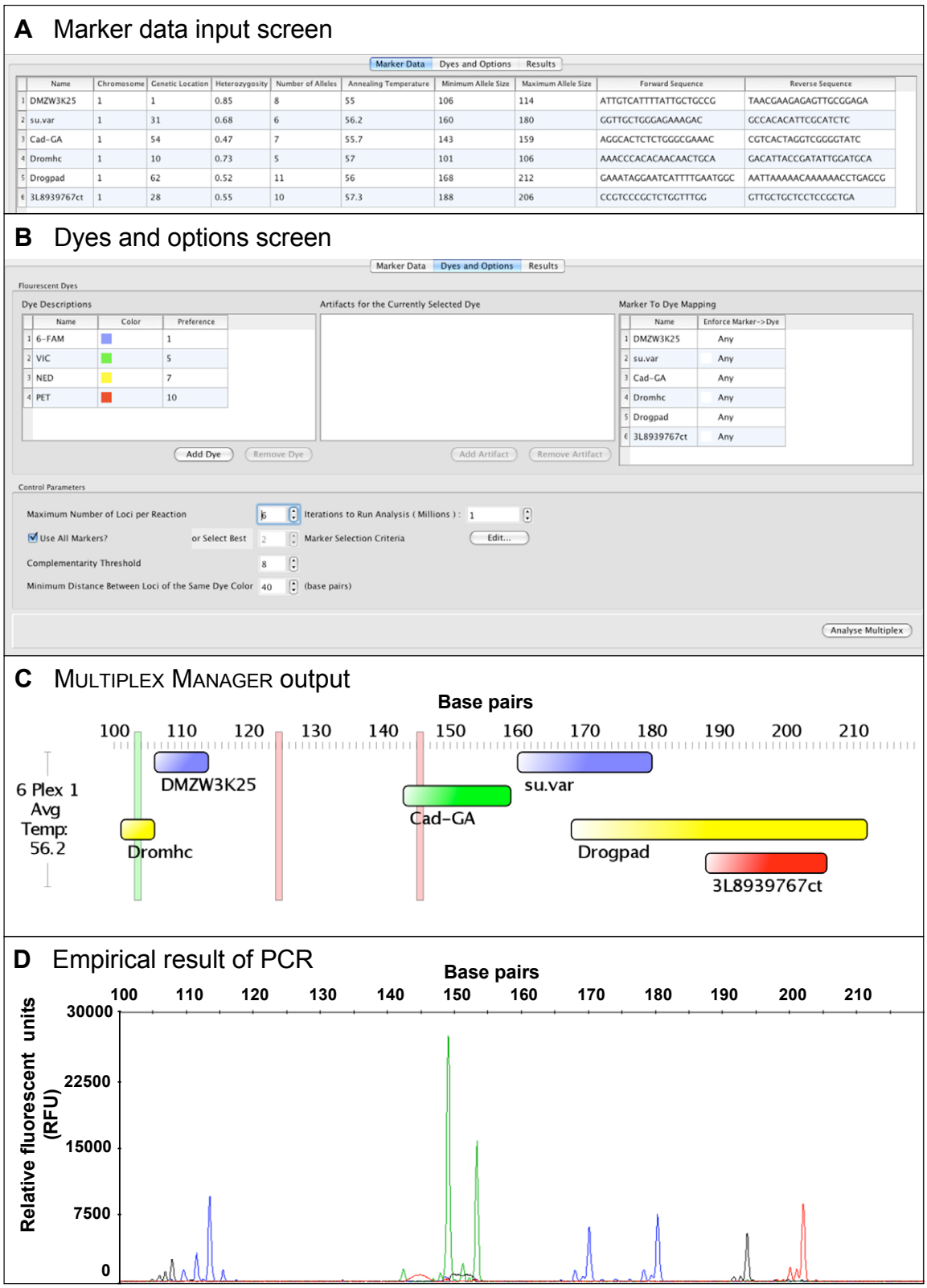

Figure 1. Worked example of how to use Multiplex Manager 1.0. (A) The user enters the primer sequences and other information into the Marker Data input window. This example uses six microsatellites from a variety of published sources [*DMZW3K25, DROMHC, DROGPAD* (9,15), *su.var* (16), *Cad-GA* (17), *3L8939767ct* (18)]. (B) The user selects the appropriate dye options and control parameters in the Dyes and Options input window. (C) Multiplex Manager combines the six microsatellites into a single multiplex reaction. (D) The user empirically tests the multiplex PCR and, if necessary, further optimizes the reaction by adjusting the concentrations of the PCR reagents. See Holleley and Sherwin (14) for reaction conditions, details about the performance of this reaction, and population genetic data.

by clicking the *Edit* option of the Control Parameters. The default marker selection criteria are: minimize the number of reactions, maximize the space between markers in the same dye, and minimize the difference in annealing temperatures of markers in the same reaction. When selecting the best *N* markers from a larger list (design scenario two), there are three additional marker selection criteria: maximizing the average heterozygosity, the number of alleles, and the standard deviation genetic location (minimizing genetic linkage) (Table 1). Multiplex Manager calculates a raw ranking score (R_i) for the enabled marker selection criteria (Table 1). The raw ranking values are later used to calculate a weighted suitability score (*SS*) (Table 1). Users must also define the primer complementarity threshold in the Control Parameters section. For a pair of primers, the complementarity score is calculated as the number of base pair matches (A-T; C-G) minus the number of mismatches (4). The default complementarity threshold score is set to 7 because empirical work has demonstrated that reactions with a score ≤7 can be successfully multiplexed (5). This threshold may be relaxed at the user's discretion.

Once the user has entered the marker data and dye preferences, and selected the appropriate control parameters, the program may be executed. The program uses a simulated annealing algorithm to sample the entire space of possible solutions (12). We have chosen a simulated annealing algorithm for this program because calculating suitability scores for all possible primer combinations quickly results in prohibitively long run times. It is critical that users specify an appropriate number of iterations for a given data set. For a moderately sized data set (≤10 markers) we recommend a minimum of 1,000,000 iterations. Larger data sets require a larger number of iterations. The program first calculates the maximum complementarity score for all primer pairs. If two markers have a complementarity score larger than the user-defined complementarity threshold, they will be not be assigned to the same multiplex reaction. The program then compares the allele size ranges of all markers and records which markers can be labeled with the same dye color. Markers are assigned to different dye colors when the allele size ranges overlap or the allele size ranges are not separated by a distance larger than the value defined in the Minimum Distance Between Loci of the Same Dye Color drop-box. The program then iteratively samples the solution space in order to determine the best combination of markers. The program creates a putative reaction by sequentially inserting markers into the reaction. The program adds a new dye to the reaction whenever allele size ranges overlap and adds a new reaction whenever the primer complementarity score for a given combination is above the threshold or the user-defined maximum number of loci per reaction has been reached. If the Select best *N* option is used, the program only inserts *N* markers into the reaction set. For each putative solution the program calculates a suitability score (*SS*) for the reaction set, weighted according to the order of the marker selection criteria specified by the user (Table 1). The weighted suitability score is calculated by multiplying the raw

Table 1. Calculating Suitability Scores with Multiplex Manager

Marker selection criteria	Algorithm to calculate raw ranking score (R_i)	Weighting (W_i)
Minimize Total Number of Reactions	Sum total number of reactions in reaction set Divide by total number of markers inserted	100,000
Maximize Spacing Between Markers In the Same Dye	For each reaction in reaction set For each dye in reaction Subtract smallest allele in dye from largest allele in dye Sum occupied size range Divide occupied size range by total size range Subtract from 1 Store result in list End End Average all results obtained above	10,000
Minimize Difference In Annealing Temperate	Calculate overall min. and max. annealing temperature For each reaction in reaction set Rescale annealing temperature over 0–1 using min./max. calculated above Calculate standard deviation of scaled values Store result in list End Average all results obtained above	1000
Maximize Average Heterozygosity	Sum heterozygosity values for all markers Divide by number of markers Subtract from 1	100
Maximize Number of Alleles	Determine maximum number of alleles in data Sum total number of alleles in reaction Divide by (number of markers × maximum number) Subtract from 1	10
Maximize Standard Deviation of Genetic Location	Determine range of genetic location values for each chromosome Rescale genetic location to be 0–1 Calculate standard deviation Store in list End Average all results obtained above	1
Overall Suitability Score (*SS*)		$\sum R_i W_i$

For each putative reaction set, Multiplex Manager calculates a suitability score (*SS*) by weighting (W_i) the raw ranking scores (R_i) of the marker selection criteria. Users can reorder the criteria and include or exclude criteria.

ranking value for each criterion (R_i range = 0–1) by a weighting factor (W_i) that decreases by an order of magnitude as one moves sequentially from most important to least important criteria in the list (Table 1). The five best solutions are stored in a list and overwritten as newer, better solutions are encountered. This process is repeated, after rearranging the markers inserted into subsequent putative reactions, for the specified number of iterations. The top rated solutions are viewed in the results window, where they can also be edited (Figure 1C). Users can alter the solution by dragging and dropping the allele size range lozenges to the desired location. Users should note that the solutions generated by Multiplex Manager do not necessarily guarantee a successful multiplex. After initial empirical tests, the reaction must be optimized for variables that are not included in the Multiplex Manager algorithm. A common problem is differential amplification of loci that can be addressed by altering the relative concentrations of primers during PCR (13); alternatively, users may decide to redesign the multiplex such that loci with a consistently low amplification yield are labeled with high quantum yield dyes. Multiplex Manager can assist in the redesign process by manipulating the Dye Preferences option or using the Force Marker to Dye option. Additionally, all loci should be tested for the presence of null alleles (14).

We conducted two tests of Multiplex Manager: first, an empirical test to demonstrate that reactions designed using this software are successful in vitro; and second, a simulation-based stress test of the software using a large data set of 408 human genetic markers, available from the Marshfield Clinic (http://research.marshfieldclinic.org/genetics/GeneticResearch/screeningsets.asp). The empirical test aimed to combine six *D. melanogaster* microsatellites from a variety of published sources [*DMZW3K25*, *DROMHC, DROGPAD* (9,15), *su.var* (16), *Cad-GA* (17), *and 3L8939767ct* (18)] into a single successful multiplex (Figure 1A). The Multiplex Manager control parameters were as follows: Maximum Number of Loci per Reaction = 6, Use All Markers? = yes, Complementarity Threshold = 8, Minimum Distance Between Loci of the Same Dye Color = 40 base pairs, and the simulated annealing algorithm was run for 1,000,000 iterations using the default marker selection criteria (Figure 1A). We tested the resulting multiplex design in 4,224 individuals using PCRs that consisted of Qiagen Multiplex 1× Master Mix, RNase-free water (Qiagen multiplex PCR kit, Cat. no. 206143; Victoria, Australia), approximately 20 ng of template DNA (19) and an equimolar concentration of primers (0.2 µM) (14). We used a step-down thermal cycling protocol as described by Holleley and Sherwin (14) to co-amplify the six primers with different annealing temperatures. PCR products were run on a 48-capillary 3730 DNA Analyzer (Applied Biosystems, Foster City, California) and fragment size analysis was conducted using GeneMapper Software Version 3.7 (Applied Biosystems). The simulation-based stress test aimed to combine the best 30 markers from the available 408 into the smallest number of multiplex PCRs. The Multiplex Manager control parameters for this test were as follows: Maximum Number of Loci per Reaction = 20, Select best *N* design scenario where *N* = 30, Complementarity Threshold = 7, Minimum Distance Between Loci of the Same Dye Color = 10 base pairs, and the simulated annealing algorithm was run for 10,000,000 iterations using the default marker selection criteria (Marshfield Clinic input files are available at www.multiplexmanager.com). Additionally, we checked that all complementarity scores calculated in Multiplex Manager were the same as those calculated in AutoDimer (4).

Results and discussion

The *D. melanogaster* multiplex PCRs amplified successfully. The locus-specific failure rate was low (0.14–1.35% in scored loci), as was the overall mis-scoring error rate (3.9%) (14). However empirical tests revealed that two loci (*DROMHC* and *DROGPAD*) were not suitable for use in population genetic analyses due to systematic deviations from Hardy-Weinberg equilibrium, most likely due to allelic dropout (14). This empirical test clearly demonstrates that Multiplex Manager can design reactions that are successful in vitro, but it also highlights how researchers must be aware of the optimization factors not addressed in Multiplex Manager (e.g., allelic dropout) and the statistical requirements of the resulting data (e.g., Hardy-Weinberg equilibrium).

For the simulation-based stress test, 30 of the 408 Marshfield Clinic markers were combined into three reactions, each reaction consisting of 9, 6, and 15 markers, respectively (Marshfield Clinic output files are available at www.multiplexmanager.com). Running this computationally intensive test for 10 million iterations using the above control parameters took only 25 min. All complementarity scores calculated by Multiplex Manager were the same as those calculated by the established program AutoDimer (4).

We have demonstrated that Multiplex Manager can combine existing genetic markers into new multiplex reactions that successfully amplify in vitro. We also showed that Multiplex Manager can handle large data sets and successfully simplify the process of designing and optimizing multiplex PCR reactions using existing marker sequences and information. Multiplex Manager 1.0 is freely available at www.multiplexmanager.com.

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